PATENT
Customer No. 22,852
Attorney Docket No. 01975-0032-00000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
DELEERSNIJDER *et al.*	Group Art Unit: 1647
Application No.: 10/030,549	Examiner: Gucker, S.
Filed: January 11, 2002	
For: HUMAN G-PROTEIN COUPLED RECEPTOR	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Fan Zhang, M.D., Ph.D., do hereby declare and say, that

1. I am a Research Scientist in the Target Discovery Group of Innogenetics, Gent, Belgium;

2. My curriculum vitae is attached as Exhibit A;

3. I am an inventor of United States Patent Application 10/030,549, entitled "Human G-Protein Coupled Receptor," filed July 17, 2000, as international application PCT/EP00/06878;

4. I have read and understood the specification of this application, including pending claims 1-33;

5. I have read and understood the Office Action regarding this application, mailed November 20, 2003, from the United States Patent and Trademark Office;

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6. I understand that claims 1-9, 11-12, 23, 25, and 26-33, have been rejected under 35 U.S.C. § 101 and 35 U.S.C. § 112 for an asserted lack of utility;

7. I have shown that the IGS1 gene is useful because it is involved in defined biological pathways, such as the dopaminergic pathway, that are related to known diseases, such as Parkinson's disease;

8. The studies described in Exhibit B were conducted under my supervision;

9. In these studies, unilateral 6-hydroxydopamine (6-OHDA) lesioned rats were used as a model of Parkinson's disease;

10. This model has been used in other studies, such as those described in Feenstra et al., Drugs of the Future, vol. 26, pp. 128-32 (2001), and is an accepted model, which has construct validity, face validity, and predictive validity, for Parkinson's disease;

11. In this model, the catecholamine neurotoxin 6-hydroxydopamine is injected into the substantia nigra on one side of the rat brain, resulting in selective lesioning of the dopaminergic cell bodies located in this area of the brain and subsequent post-synaptic adaptation that causes supersensitivity of D2 receptors;

12. Such unilateral lesioning by 6-OHDA injection results in asymmetric behavior of the treated animals, such as moving in circles;

13. The rats that received the 6-OHDA injections in this study showed convincing clinical and biochemical evidence that they were unilaterally lesioned;

14. In situ hybridization (ISH) of 6-OHDA lesioned rats brains was performed using a nucleic acid probe specific for IGS1 mRNA, as described in the Materials and Methods section of Exhibit B at 2-7;

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15. In one experiment, statistically significant decreases in the ISH signal for IGS1 mRNA were found in the following regions, when the lesioned side was compared to the intact side:

nucleus accumbens	9.3%, p=0.0462
dorsomedial caudate putamen	36.8%, p=0.0003
ventromedial caudate-putamen	33.6%, p=0.0007

16. No significant difference in the ISH signal for IGS1 mRNA on the lesioned side compared to the intact side was seen in the dorsolateral caudate-putamen (-3.3%, p=0.6912) or ventrolateral caudate-putamen (0.8%, p=0.9383);

17. In a second experiment, similar results were found, with the following decreases in ISH signal of IGS1 mRNA observed in the lesioned side, when compared to the intact side:

nucleus accumbens	9.5%, p=0.0125
dorsomedial caudate putamen	35.8%, p=0.0001
ventromedial cuadate putamen	40.0%, p=0.0002

18. In the second experiment, again no difference was observed in the ISH signal for IGS1 mRNA on the lesioned side compared to the intact side in the dorsolateral caudate-putamen (3.0%, p=0.7830) or ventrolateral caudate-putamen (0.8%, p=0.9574);

19. A Bonferoni test showed that the differences in the dorsomedial and ventromedial striatal areas were statistically significant in all experiments;

20. These results are provided in Figures 7 and 8 of Exhibit B;

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21. These results indicate that IGS1 mRNA expression is involved in the dopaminergic pathway, is regulated by midbrain dopamine innervation, and that the cellular compartments that express IGS1 mRNA are directly or indirectly involved in the information processing of dopaminergic afferents to the striatum;

22. It is known that the dorsal striatum (caudate striatum) receives dopaminergic projections from the substantia nigra pars compacta (SNc), which is implicated in motor function;

23. It is known that SNc is markedly affected by Parkinson's disease;

24. The pronounced decrease in IGS1 mRNA in the medial caudate-putamen upon lesioning, shown in the results of this study, indicates that IGS1 has a biological role in motor function and Parkinson's disease;

25. Therefore, it is likely the determination of the level of IGS1 in the brain would have a significant role in diagnosing Parkinson's disease;

26. It is also likely that agonists and antagonists of IGS1 should have a significant role in treating and preventing Parkinson's disease.

27. Normal expression of IGS1 mRNA was also studied by ISH;

28. High levels of IGS1 mRNA were observed in the amygdala and striatum, particularly in the ventral striatum (accumbens nucleus, olfactory tubercle, and ventral medial caudate-putamen), as compared to the dorsal counterpart (dorsal caudate-putamen) (Exhibit B, Figure 10);

29. It is known that the amygdala is part of the limbic system and that the ventral striatum (nucleus accumbens mainly) receives innervation from the ventral tegmental areas (VTA), which is implicated in limbic function;

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30. Limbic function is involved in eating and drinking habits, emotional changes, such as fear, anxiety, rage, and aggressive behavior;

31. This study shows that IGS1 is normally expressed in regions of the brain associated with limbic function;

32. Therefore, IGS1 may have a significant role in diagnosing psychiatric diseases affecting limbic function;

33. Agonists and antagonists of IGS1 may also have a significant role in treating and preventing psychiatric diseases characterized by limbic dysfunction.

The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing therefrom.

By: _____ Dated: Feb. 18, 2004
Fan Zhang, M.D., Ph.D.

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EXHIBIT A

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General

Name and Family Name:	Fan Zhang
Date of Birth:	November 1, 1962
Place of birth:	Shanghai, China
Sex:	Female
Marital Status:	Single
Nationality:	Belgian

Employment History

Since 9/1998, working as a research scientist in Target Discovery Group in Innogenetics, Gent, Belgium.

Education

1997-1998	**University of British Columbia**	Vancouver	**Post-doctor** in Neuroscience
1991-1996	**Universite Libre de Bruxelles**	Brussels	**Ph.D** in Neuroscience
1989-1991	**Vrije Universiteit Brussel**	Brussels	**Master** in Med. & Pharm. Res.
1985-1989	**Shanghai Mental Health Center**	Shanghai	Specialization in **Psychiatry**
1980-1985	**Shanghai Second Medical School**	Shanghai	**Doctor in medicine**

Grants Received

1997-1998	Medical Research Council Fellowship, Vancouver, Canada
1991-1996	Queen Elizabeth Foundation Fellowship, Brussels, Belgium
1990-1991	Research assistant, Vrije Universiteit Brussel, Brussels, Belgium
1989-1990	Diabetes Research Fellowship, Brussels, Belgium

Publications

•Articles:

1. **ZHANG F.**: Islet amyloid polypeptide (IAPP) in the human fetal pancreas: an immunocytochemical study. Thesis for obtaining a degree entitled "Master in Medical and Pharmaceutical Research" in Vrije Universiteit Brussel, 48 pages, 1991.

2. IN T'VELD P.A., **ZHANG F.**, MADSEN O.D. and KLÖPPEL G.: Islet amyloid polypeptide immunoreactivity in the human fetal pancreas. *Diabetologia,* 35: 272-276, 1992.

3. MAILLEUX P., **ZHANG F.** and VANDERHAEGHEN J.-J.: The dopamine D1 receptor antagonist SCH-23390 decreases the mRNA levels of the transcription factor zif268 (krox-24) in the adult rat striatum-an in situ hybridization study. *Neuroscience Letters,* 147: 182-184, 1992.

4. **ZHANG F.**, HALLEUX P., ARCKENS L., VANDUFFEL W., VAN BREE L., MAILLEUX P., VANDESANDE F., ORBAN G.A., VANDERHAEGHEN J.-J.: Distribution of immediate early gene Zif-268, c-fos, c-jun and jun-D mRNAs in the adult cat with special references to brain region related to vision. *Neuroscience Letters,* 176: 137-141, 1994.

5. JACOBS O., VAN BREE L., MAILLEUX P., **ZHANG F.**, SCHIFFMANN S.N., HALLEUX P., ALBALA N. and VANDERHAEGHEN J.-J.: Homolateral cerebrocortical increase of immediate early gene and neurotransmitter messenger RNAs after minimal cortical lesion. Blockade by N-methyl-D-aspartate antagonist. *Neuroscience,* 59: 827-836, 1994.

6. **ZHANG F.**, VANDUFFEL W., SCHIFFMANN S.N., ORBAN G.A., VANDERHAEGHEN J.-J.: Decrease of c-fos and zif-268 and increase of c-jun mRNAs in the cat areas 17, 18 and 19 following complete visual deafferentation. *Eur. J. Neuroscience,* 7: 1292-1296, 1995.

7. **ZHANG F.**, VAN BREE L., ALBALA N., VERSLYPE M., VANDERHAEGHEN J.-J.: NMDA receptor antagonist MK-801 down-regulates rat striatal proenkephalin and protachykinin mRNAs. *Neurochem. Int.,* 28: 189-192, 1995.

8. ARCKENS L., **ZHANG F.**, VANDUFFEL W., MAILLEUX P., VANDERHAEGHEN J.-J., ORBAN G.A. and VANDESANDE F.: Localization of the two protein kinase Cβ-mRNA subtypes in cat visual system. *J. Chemical Neuroanatomy,* 8: 117-124, 1995.

9. VAN BREE L., **ZHANG F.**, SCHIFFMANN S.N., HALLEUX P., MAILLEUX P., VANDERHAEGHEN J.-J.: Homolateral cerebrocortical changes in neuropeptide and receptor expression after minimal cortical infarction. *Neuroscience,* 69: 847-858, 1995.

10. **ZHANG F.**, Modulation of gene expression in the central nervous system. Thesis for obtaining a degree entitled " thèse d'agrégation l'enseignement supérieur en neurosciences " in Université Libre de Bruxelles, 101 pages, 1996.

•Communications in Conferences

1. **ZHANG F.**, MADSEN O., KLÖPPEL G. and IN T'VELD P.A.: Islet amyloid polypeptide (IAPP) is a marker for a subpopulation of human fetal B-Cells. Abstract of the *27th Annual Meeting of the European Association for the Study of Diabetes,* Dublin, 10-14 September 1991, 416, A105, 1991.

2. VANDESANDE F., **ZHANG F.**, VANDUFFEL W., MAILLEUX P., ARCKENS L., VANDENBUSSCHE E., VANDERHAEGHEN J.-J. and ORBAN G.A.: Visual deafferentation induces modifications of Immediate Early Genes (IEG) and Neurotransmitters gene expression in the adult cat brain. Abstract of the *23rd Annual Meeting, Society for Neuroscience,* Washington D.C., November 7-12, 1993.

3. **ZHANG F.**, HALLEUX P., VANBREE L., ALBALA N., VERSLYPE M., MAILLEUX P., VANDUFFEL W., ARCKENS L. and VANDERHAEGHEN J.-J.: Distribution of immediate early gene Zif-268, c-fos, c-jun and jun-D mRNA in the adult cat with special references to brain region related to vision. Abstract of the *2nd Annual Meeting UIAP/PAI,* Louvain, December 21, 1993.

4. VAN BREE L., **ZHANG F.**, MAILLEUX P. and VANDERHAEGHEN J.-J.: NMDA receptors mediate diffuse homolateral cerebrocortical changes in gene expression following a minimal cortical lesion. Abstract of the *XIXth C.I.N.P. Congress, Collegium Internationale Neuro-Psychopharmacologicum,* Washington, U.S.A., June 27-July 1, 1994.

5. VAN BREE L., **ZHANG F.**, MAILLEUX P. and VANDERHAEGHEN J.-J.: A minimal cerebro-cortical lesion produces extensive changes in immediate early gene and neurotransmitter gene expression in the homolateral cortical hemisphere. Abstract of the *17th Annual Meeting of the European Neuroscience Association in association with the 26th Annual Meeting of the European Brain and Behaviour Society and the Meeting of the Austrian Neuroscience Association,* Austria Center Vienna, Austria, September 4-8, 1994.

6. **ZHANG F.**, VANDUFFEL W., ARCKENS L., SCHIFFMANN S.N., VERSLYPE M., ALBALA N., VANDESANDE F., ORBAN G.A. and VANDERHAEGHEN J.-J.: Decrease of Zif-268 and c-fos and increase of c-jun mRNAs in the cat areas 17, 18 and 19 following complete visual deafferentation. Abstract de la *Fondation Médicale Reine Elisabeth et Société Belge de Neurologie,* séance consacrée aux Neurosciences, Bruxelles, le 5 novembre 1994.

7. **ZHANG F.**, VERSLYPE M., Conreur J.-L. and VANDERHAEGHEN J.-J.: Early gene expression in the visual cortex afer learning and in split brain. Abstract of the 4th *Annual Meeting UIAP/PAI,* Louvain, December 13, 1995.

8. **ZHANG F.**, FIBIGER H.C., ZIS A. P., Differential effects of two doses of ECS on c-fos expression in the hypothalamus. Abstract of the *53rd Annual Convention of Society of Biological Psychiatry,* Toronto, Canada, May 27-31, 1998.

EXHIBIT B

BEST AVAILABLE IMAGES

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Annex 2

Modulation of IGS1 expression in a rat model of Parkinson's disease

INTRODUCTION

Using Northern blot analysis we have demonstrated that a novel G-protein coupled receptor (GPCR) named IGS1 is exclusively expressed in a restricted area of the human CNS. Within CNS, high IGS1 mRNA levels were found in caudate nucleus and putamen. These brain areas are part of the basal ganglia network. This network has been at the center of intensive research efforts since the discovery that the hallmark of Parkinson's disease - a reduction of striatal dopamine - is the result of neuronal degeneration in midbrain areas, namely in substantia nigra. The finding of high IGS1 mRNA expression in these brain areas prompted us to investigate the potential involvement of this gene in the dopaminergic pathway and eventually in Parkinson's disease.

Unilateral 6-hydroxydopamine (6-OHDA) lesioned rats, as described originally by Ungerstedt (Eur. J Pharmacol 5:107-110; 1968) are a widely used model system to study Parkinson's disease and to assess potential anti-Parkinsonian efficacy of compounds. Briefly, the catecholamine neurotoxin 6-hydroxydopamine is injected routinely into the substantia nigra on one side, resulting in selective lesioning of the dopaminergic cell bodies located in this brain area. Subsequently, dopamine-containing projections to the ipsilateral striatum degenerate over a period of 10 days resulting in dopamine depletion in this brain area and post-synaptic adaptation occurs, i.e. dopaminergic D2 receptors become supersensitive. Thus, using D2 receptor agonists will become more effective at the super-sensitive side than on the contralateral side resulting in asymmetric behavior, i.e. animals start moving in circles. Its number of rotations indicate a) the completeness of the lesion, and b) can be used as estimate for anti-Parkinsonian efficacy for Parkinson's disease. This model has been validated in a number of key papers, using a variety of dopamine D2 receptor agonists (e.g. see Feenstra et al., Drugs of the future 26: 128-132; 2001). Thus,

unilateral 6-OHDA lesions of the rat substantia nigra has construct validity, face validity and predictive validity for Parkinson's disease.

In addition, this model yields neurochemical data on adaptive changes in striatal tissue. Thus, changes in striatal dynorphin levels have been associated with risk for dystonia. Therefore, analysis of adaptive changes in expression may point to novel targets that may be able to couteract part of the adaptive changes, induced by progressive depletion of dopamine in the striatum. In this respect, ligands for IGS1 may be effective in treating the hypokinesia, dystonia, associated with Parkinson's disease. Alternatively, ligands for IGS1 may be effective in counteracting some of the iatrogenic effects that current therapy has, e.g. reducing the "on-off" phenomena that occur after prolonged treatment with anti-Parkinsonian drugs.

We decided to study the modulation of IGS1 mRNA expression by the 6-OHDA lesions in rats via in situ hybridization (ISH) in order to study its role in the dopaminergic pathway and its potential involvement in adaptive changes induced by dopamine depletion.

EXPERIMENTAL PROCEDURES.

Generation and use of 6-OHDA lesioned rats.

6-Hydroxydopamine lesioning

Male Wistar rats (Harlan The Netherlands, Wistar Unilever (HsdCpb:WU)) weighing 250 – 350gr are routinely used. Animals are pretreated with DMI one hour before operation to protect noradrenergic neurons and subsequently anesthetised using 3% halothaan + 0.8 l/min N2O + 0.8 l/min O2. Lesioning of the left substantia nigra is done by stereotactic placement of a needle into the substantia nigra pars reticulata after which 3 µl 6-OHDA solution (3.33 mg/ml in phosphate—buffered saline containing 0.2 mg/ml vitamine C) is infused at a rate of 0.75 µl/min. After infusion, the needle is kept in place for 4 min to allow proper difusion of the neurotoxin before slowly withdrawing the needle.

Turning behavior

Typically, 14 days after lesioning, rats are habituated to the rotameter (TSE systems), linked to a PC which records the number of turns the animal makes. Animals are tethered to a rotating sensor and are placed in plastic bowls (57x55x52 cm; 8 units in parallel). Measurements are routinely made for 5 min. Standardisation of measurements is routinely done by administering apomorphin or amphetamine to induce contralateral turning or ipsilateral turning respectively. Acceptable limits to lesion quality is that animals perform at

least 20 turns per 5 min. Animals that meet these criteria can be used for screening purposes or brains can be processed for neurochemical measurements such as immunocytochemistry or in situ hybridisation.

The 6-OHDA turning rat model has been validated as a model for Parkinson's disease as it contains face validity, construct validity and predictive validity for symptomatic treatment in Parkinson's disease (see also Ungerstedt U, 1971, Postsynaptic super-sensitivity after 6-hydroxy-dopamine-induced degeneration of the nigro-striatal dopamine system, Acta Physiol Scand 82 (Suppl.367): 69-93, and Ungerstedt U, 1971, Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behavior, (Suppl.367): 49-68).

In addition, this animal model has been widely used to identify adaptive mechanisms that change upon dopamine denervation and may yield other approaches for treatment of Parkinson's disease. Several targets have been evaluated since, including muscarinic receptors, μ-opiate receptors and adenosine A2a receptors.

Preparation of tissue sections.

Normal rats Four male Wistar rats were used in the study to map the normal distribution of IGS1 mRNA in the brain. All animals were kept in a 12/12 hours light/dark cycle. They were allowed to recover from transportation for one week prior to sacrification. Rats were decapitated, and their brains were dissected out, immediately frozen in 2-methylbutane and cooled on dry ice. The brains were stored at –70°C until use. Tissues were then sliced coronally (n=3 brains) or saggitally (n=1 brain) into 15μm thick sections using a cryomicrotome (Leica CM3050). Sections were thaw-mounted on glass slides coated with 0.1% poly-L-lysine and stored at –20°C.

6-OHDA lesioned rats. B rains (rats n° 2, 5 & 6) were sectioned coronally into 15μm thick sections using a cryomicrotome (Leica CM3050). Sections were thaw-mounted on glass slides coated with 0.1% poly-L-lysine and stored at –20°C.

Preparation of IGS1 riboprobes. Riboprobes were prepared by in vitro transcription (IVT) of IGS1 specific PCR amplicons to generate IGS1 antisense probes. The sequence of the T7 RNA polymerase promoter had been appended at the 5' end of the antisense primers used to generate rat IGS1 specific amplicons (from the rat IGS1 containing template

plasmid IGS1.2). Following PCR primers were used to generate 2 different (non-overlapping) IGS1 specific amplicons:

Amplicon	Length	Primers	Sequence
F7R7	203 bp	Forward:IP14554	5'-ATTTACGTGGGCGAAGATGAC-3'
		Reverse:IP14555	5'GCCAGTAATACGACTCACTATAGGG AGAGCCCAGAGACAGCACATAGGAG-3'
F8R8	254 bp	Forward:IP14556	5'-GACGAGAATGAGTTTGAGGAC-3'
		Reverse:IP14557	5'GCCAGTAATACGACTCACTATAGGG AGAGGTTGCACTGATTGACATCTCT-3'

PCR reactions were run in a 50 µl volume and contained 20 pmol each of the forward and reverse primers, 0.8 µl dNTPs (25mM each), 5µl 10 x reaction buffer, 2.5U AmpliTaq Gold polymerase and 1.65 ng rat IGS1 template (IGS1.2 plasmid) was added. PCR reaction tubes were then subjected to 25 cycles of denaturation (94°C for 30s), annealing (55°C for 30s) and extension (72°C for 1min). There was a final extension at 72°C for 7 min. The PCR products were analyzed via agarose gel electrophoresis after ethidium bromide staining. Under the above conditions, PCR reactions yielded a clear band of predicted length.

In vitro transcription reactions to generate ^{33}P labeled riboprobes were carried out using the SP6/T7 Transcription kit (Boehringer cat. no. 999 644). The in vitro transcription reaction contained 2 µl of the unpurified PCR amplicons, 3 µl ATP/GTP/CTP mix (2.5 mM each), 2 µl 10x transcription buffer, 5 µl [^{33}P]-UTP (>2500 Ci/mmol, 20 mCi/ml), 1 µl RNAse inhibitor, 6 µl DEPC treated water, 1 µl T7 RNA polymerase (10 units/µl) and was incubated at 37°C for 2h. The template DNA was then digested by adding 20 units of RNAse free DNAse I for 15 min at 37°C. The reaction was stopped by adding 2 µl of 0.2 M EDTA. The synthesized riboprobe was purified using Quick Spin™ columns (Boehringer cat no. 1 273 990). The amount of the probe that was produced and its specific activity were determined from the degree of incorporation of the radioactive nucleotide (as calculated from the amount of counts from an aliquot of the IVT reaction products that remained bound to DE81™ filters (Whatman) after washing versus the total counts before washing). Specific activity of the riboprobes was in the range of $8.4x10^7$ to $1.7x10^9$ cpm/µg. The size and purity of the riboprobes was verified via denaturing agarose gel electrophoresis. Single bands of expected length were observed.

To produce large amounts of cold riboprobe to be used in the control hybridizations, in vitro transcription reactions were done using the T7-Megashortscript™ kit (Ambion, cat. no. 1354). Briefly, 2 µl of the unpurified PCR amplicon, 8 µl ATP/ CTP/ GTP/ UTP mix (75 mM each), 2 µl 10x transcription buffer, 6 µl DEPC treated water, and 2 µl T7 Megashortscript Enzyme Mix, were incubated at 37°C for 4 hour. The template DNA was then digested by incubating the mixture with 2 units of RNAse free DNAseI at 37°C for 15 min. This reaction was stopped by heating at 95°C for 5 min. Probes were purified using Quick Spin™ columns. The yield was in the range of 10.4-12.8 µg/ 20 µl reaction. The size and purity of the riboprobes were verified via denaturing RNA gel electrophoresis. Single bands of expected length were observed.

In situ hybridization using IGS1 riboprobes. Sections were fixed in 4% paraformaldehyde in 0.01 M sodium phosphate buffer (PBS, pH 7.4) at 4°C for 30 min. Sections were then rinsed in PBS, followed by dehydration in graded series of ethanol (70%, 90% and 100%), delipidation in chloroform for 5 min and then rinsed once in 100% ethanol for 1 min. The sections were incubated with hybridization solution (200 µl/slide). The hybridization solution contained 20mM phosphate buffer, 50% formamide, 4xSSC, 1 x Denhardt's solution, 10% dextran sulphate, 1% sarcosyl and $5x10^5$ cpm/slide of the ^{33}P-labeled riboprobe. Hybridization reactions were carried out at 50°C overnight. Post-hybridization washings were either done under stringent conditions or at reduced stringency but including RNAse treatment. Stringent washing demonstrated the same specific labeling pattern as did less stringent washes including RNAse treatment but exhibited more intense hybridization signals. Thus except for the initial experiments aimed at testing the specificity of the IGS1 riboprobes stringent washing was applied in all other experiments.

Reduced stringency washing including RNAse treatment. After hybridization, the sections were washed once in 0.1x SSC at 50°C for 10 min. then in 2x SSC at RT for 10 min, followed by RNAse A (5 µg/ml in PBS) treatment for 15 min at 37°C, a further wash in 0.1xSSC at 37°C for 30 min and a final wash in 2xSSC for 5 min at RT.

Stringent washing: After hybridization, sections were washed once in 2x SSC containing 50% formamide at 50°C for 30 min. followed by 2 serial washes in 0.1 x SSC at 62°C for 90 min. The slides were dehydrated, air dried and then autoradiographed first using phosphor-imaging screens, then using X-ray films (Amersham βmax for the expression analysis in normal rat brains and Kodak BioMax MR film for the 6-OHDA lesion study) and finally via dipping in autoradiographic emulsion (Amersham Hypercoat emulsion LM-1).

Control hybridization reactions were performed by co-hybridization with more than 100 fold excess of either a corresponding (= identical sequence as the radio-labeled probe)

or a non-corresponding cold anti-sense riboprobe. Non-corresponding probes were chosen from the rat IGS1 sequence but were non-overlapping with the radio-labeled probe (i.e. the excess cold F8R8 probe was used as control for the radio-labeled F7R7 probe, and vice versa).

In situ hybridisation using a proenkephalin oligonucleotide probe. A proenkepahalin oligonucleotide anti-sense probe was labelled at the 3' end with ^{35}S-dATP using the terminal transferase enzyme (Boehringer cat 220 582). Briefly 80 ng of the oligonucleotide were incubated with 50 U terminal transferase, 50 µCi ^{35}S-dATP [> 1,000 Ci/mmol] and 4 µl reaction buffer (supplied with the enzyme) in a total volume of 20 µl for 60 min at 37°C. The radiolabeled probe was purified using a nucleic acid purification cartridge (NENSORB 20, Life Technologies). The specific activity of the radio-labeled probe was 5 x 10^7 cpm/µg.

After fixation in 4% paraformaldehyde for 30 min, sections were incubated overnight at 37°C with 8 x 10^5 cpm per section of the ^{35}S labelled proenkephalin probe in hybridization buffer (200 µl/ section). The hybridization buffer contained 50% formamide (Fluka), 4 x SSC, 1 x Denhardt's solution, 1% sarcosyl, 10% dextran sulfate, yeast tRNA at 275 µg/ml, salmon sperm DNA at 100 µg/ml, and 60 mM dithiothreitol. After hybridization, the sections were rinsed 4 x 15 min in 1 x SSC at 42°C. Sections were covered first by a multipurpose phosphor storage screen for 4 hours to obtain a quick impression and then covered by X-ray film (Kodak Bio Max) for 7 days to obtain a higher resolution autoradiographic image. Autoradiography at cellular resolution was performed by dipping the sections in autoradiographic emulsion (Amersham Hypercoat emulsion LM-1).

Quantification of the ISH signals. Using a Nikkon D1™ digital camera, images from autoradiographic films were captured on a light box under standard conditions of aperture (F3.8), shutter speed (1/500) and 'film' sensitivity (200ASA). The intensity- and contrast-balancing tools of the Nikkon Capture™ software were used to obtain optimal imaging. The same setting was then used in all the images to have an equalized background. Digitized images were then quantified.

Quantification was done using the Scion Image ™ (release β4.0.2) software. The area to be quantified was indicated (drawn) manually on the screen (Fig.1 & 3). The computer measured the mean density of the defined area (= sum of the gray values of all the pixels of the selected area divided by the total number of pixels in this area). A background area adjacent to the section was also quantified and this value was subtracted from each measurement (Fig. 1).

For proenkephalin mRNA quantification, the entire caudate-putamen area of each hemisphere was quantified (Fig. 1). The measurements from the same hemisphere of each rat (3 sections/rat) were combined and the mean value was calculated. The mean value obtained from the lesioned side was compared with that from the intact side in each rat using the two-tailed paired Student's t test.

For IGS1 mRNA quantification, 3 consecutive sections of each rat brain were randomly selected within the striatal area. Two independent ISH experiments (3 sections / experiment) were done to evaluate the reproducibility of the results (Fig. 2). In each independent experiment, all sections were processed in the same run. Because of the heterogeneous expression pattern of IGS1 in striatum, hybridization intensities were quantified in the subareas of this brain region: accumbens nucleus, dorsomedial, ventromedial, dorsolateral and ventrolateral caudate-putamen (Fig. 2 & 3). The measurements (from all 3 rats) of each defined brain area of the same side were combined and the mean value was calculated. The mean of the lesioned side was compared to that of the intact side in a given area. The two-tailed paired Student's t test and the Bonferoni test were used for statistical analysis.

RESULTS.

Normal distribution of IGS1 mRNA. Specific ISH signals for IGS1 mRNA were observed in rat brain sections. The ISH signals were completely abolished by the presence of excess corresponding cold probe, but remained unchanged in the presence of excess non-corresponding cold probe. Moreover, 2 non-overlapping probes (F7R7 and F8R8) generated an identical labeling pattern and signal intensity in independent experiments, indicating the specificity and reproducibility of the ISH signal (data not shown). The F7R7 riboprobe was used in all experiments described in the following paragraphs.

In the brain areas investigated, IGS1 ISH signals were observed in the cerebral cortex, caudate-putamen, accumbens nucleus, olfactory tubercle, amygdaloid complex, thalamus, hypothalamus, hippocampus, and cerebellum. High IGS1 mRNA expression was found in the striatum, particularly in the ventral striatum, amydala, septum, and arcuate nucleus of hypothalamus, as well as in the cerebellum (Fig. 10).

In the striatum, high level of IGS1 mRNA ISH signals was observed. The ISH labeling was higher in the ventral striatum (accumbens nucleus, olfactory tubercle and ventral medial caudate-putamen) than in its dorsal counterpart (dorsal caudate-putamen). At the cellular level, ISH signals were observed in medium-sized striatal neurons,

presumably dopaminergic neurons, in both dorsal and ventral striatum (Fig. 4). A larger number of labelled neurons (and with higher labeling intensity) were observed in the ventral striatum than in the dorsal striatum. No labeling could be seen on large neurons.

Proenkephalin mRNA in the striatum of the 6-OHDA lesioned rats. The proenkephalin oligonucleotide probe demonstrated a labeling pattern that was identical to that described in published reports (Fallon J.H. et al. J. Comp. Neurol. 249:293-336; 1986) In the brain areas investigated, proenkephalin ISH signals were predominantly present in the dorsal (caudate-putamen) and ventral (accumbens nucleus and olfactory tubercles) striatum, and faintly in the cerebral cortex (Fig. 1), indicating that the probe is specific.

In all three 6-OHDA lesioned rats (no. 2, 5 and 6) studied, a marked increase of proenkephalin mRNA level was found in the caudate-putamen ipsilateral to the lesion at the macroscopical as well as at the cellular level (Fig 1). Under the microscope, the silver grains were found clustered on top of neurons. As expected, the striatal neurons were much more heavily labeled in the lesioned side than in the intact side (Fig 1).

Quantification of the proenkepahalin ISH signals (Fig. 5 & 6) demonstrated a 53% ($p=0.026$), 37% ($p=0.006$) and 59% ($p=0.003$) increase of proenkephalin mRNA levels in the lesioned sides of striatum of rats n° 2, 5 and 6 respectively.

IGS1 mRNA in the striatum of the 6-OHDA lesioned rats. A slight decrease of the IGS1 mRNA ISH signal could be seen on film autoradiographs in the medial caudate-putamen ipsilateral to the lesion (Fig. 7). A small, but statistically significant decrease was observed in the nucleus accumbens (9.3%, $p=0.0462$) and in the dorsomedial (36.8%, $p=0.0003$), and ventromedial (33.6%, $p=0.0007$) caudate-putamen of the lesioned side as compared to the intact side. No significant difference could be found in the dorsolateral (-3.3%, $p=0.6912$) and ventrolateral (0.8%, $p=0.9383$) sides of the caudate-putamen (Fig. 8 & Fig. 9).

These data were confirmed by a second independent ISH experiment. A significant decrease was found in the nucleus accumbens (9.5%, $p=0.0125$), in the dorsomedial (35.8%, $p=0.0001$) and ventromedial (40.0%, $p=0.0002$) part of caudate-putamen of the lesioned side, and no difference in the dorsolateral (3.0%, $p=0.7830$) and ventrolateral (0.8%, $p=0.9574$) areas of the caudate-putamen (Fig. 8 & 9).

The Bonferoni test showed that only the differences in the dorsomedial ($p=0.003$ and $p=0.001$ in experiments 1 and 2 respectively) and ventromedial striatal areas ($p=0.007$ and $p=0.002$ in experiments 1 and 2 respectively) was statistically significant in all experiments.

DISCUSSION.

The ISH hybridization data obtained on normal rat brain are in agreement with Northern blot data obtained on human tissue. In both studies high expression levels of IGS1 mRNA were observed in the caudate-putamen. Moreover ISH studies at the cellular level demonstrated that IGS1 labeling is mainly, if not solely, present in the medium-sized striatal neurons. Since over 90% of the striatal medium-sized neurons are dopaminergic, we decided to study modulation of IGS1 levels by 6-OHDA induced lesions in order to investigate the involvement of IGS1 in dopaminergic pathways.

Validation of the rat model after unilateral injection of 6-OHDA in the nigrastriatal projection can be done either clinically or biochemically. A depletion of striatal dopamine will result in supersensitivity of dopamine receptors. Because the midbrain dopaminergic projections from the substantia nigra to the striatum are ipsilateral in rat and man, this leads to a contralateral turning behavior when the rats are challenged with the dopamine agonist apormorphine. It is known in the literature that dopamine inhibits striatal proenkephalin expression at the basal condition. The depletion of dopamine thus disinhibits or increases the proenkephalin expression in the lesioned side of striatum. The 3 rats used in this study met rotation criteria as described above and showed convincing clinical and biochemical evidence that they were well lesioned unilaterally.

We demonstrated a small, but statistically significant, decrease of IGS1 mRNA levels in the medial striatum ipsilateral to the lesion as compared to the corresponding contralateral area. The observed decrease was consistent among all 3 rats and could be observed in 2 independent experiments. Moreover, the decrease in the medial striatum was significant as evaluated with the stringent Bonferoni test, ruling out a possible statistical error resulting from multiple comparison.

These data suggest that striatal IGS1 mRNA expression is regulated by midbrain dopamine innervation. Moreover, these data indicate that the cellular compartments that express IGS1 mRNA are directly or indirectly involved in the information processing of dopaminergic afferents to the striatum.

It is known that the dorsal striatum (caudate-putamen) receives dopaminergic projections from substantia nigra pars compacta (SNc), whereas the ventral striatum (nucleus accumbens mainly) receives innervation from ventral tegmental areas (VTA). The former is implicated in the control of motor function, while the latter is involved in limbic functions. Indeed, the SNc is markedly affected in Parkinson's patients. The more pronounced decrease in IGS1 mRNA in the medial caudate-putamen than in the nucleus

accumbens suggests its involvement in the control of motor function, and possibly Parkinson's disease.

The normal distribution of IGS1 mRNA demonstrated that it is highly expressed in the limbic systems, such as amydala, hippocampus, septum, as well as hypothalamus. It is known in both animal and human studies that stimulation or lesion in certain areas in the limbic system may induce changes in eating and drinking habits, emotional changes such as fear, anxiety, rage and aggressive behavior, as well as changes in sexual behavior. This is particularly interesting since typical depression symptoms are composed of mood, eating habit, and sexual behavior changes. It suggests therefore that IGS1 is likely to take part in the control of these behaviors and eventually might be a potential target for the treatment of psychiatric diseases.

The IGS1 receptor distribution resembles that of the adenosine A2a receptors. Also this receptor is located in striatum of rats and man. Moreover, it has been established that A2a receptor agonists can inhibit the release of the neurotransmitter GABA from striatal slices, whereas using the same preparation, A2a receptor agonists can stimulate the release of the neurotransmitter acertylcholine. Moreover, chronic exposure of rats to a A2a receptor antagonist reduces the expression in striatum of the neuropeptide enkephalin.

Using unilaterally 6-hydroxydopamine-lesioned rats, contralateral rotation behavior can be induced by dopamine D2 agonists. Interestingly, A2a receptor agonists reduce the number of contralateral rotations.

Also, with unilateral 6-OHDA lesions, expression of the neuropeptide enkephaline goes up as shown in our own studies as well. This increase can be prevented by exposure to adenosine A2a receptor antagonists.

Therefore, we argue that as IGS1 has a distribution in striatal tissue that closely resembles that of adenosine A2a receptors, it is conceivable that the use of suitable receptor agonists or antagonists can elicit the same promising effects as has currently been identified for A2a receptor antagonists.

These claims can be extended by the notion that striatal dynorphin expression is indicative for the amount of dystonia that is observed in animal models, predictive for human therapeutic intervention. As striatal dynorphin expression is also subject to dopaminergic input, it is expected that suitable agonists or antagonists for IGS1 receptors may be beneficial in regulating striatal dynorphin expression in such a way that the risk for development of dystonia (a symptom associated with Parkinson's disease) can be severely reduced.

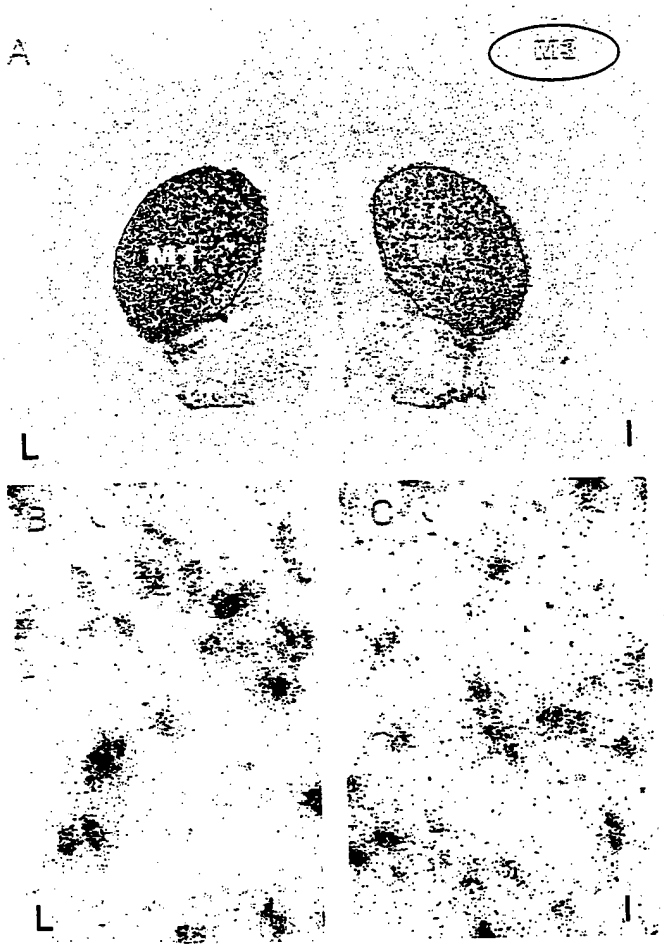


Fig. 1 Delineation of areas that were used for proenkephalin mRNA quantification in the striatum of unilaterally 6-OHDA lesioned rats. Gray values were measured in the defined areas (M1, M2, & M3). An increase of proenkephalin mRNA is visible in the lesioned side of striatum (L) compared to the intact side (I) at both macroscopical (A) and microscopical (B,C) levels.

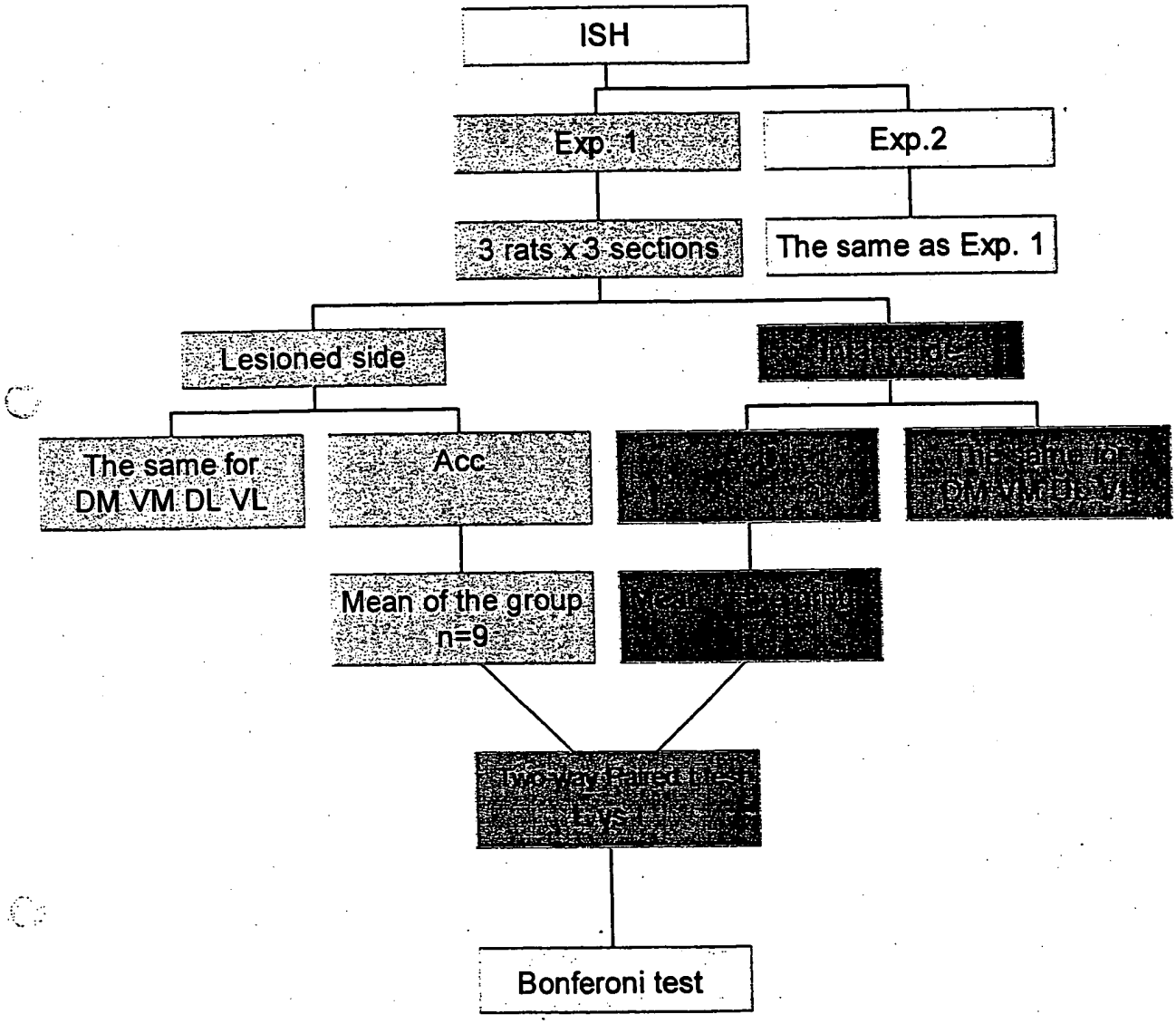


Fig. 2 Outline of protocol used to determine differential expression of IGS1 mRNA in rat striatal subregions following unilateral 6-OHDA lesion.
Abbreviations: Acc, accumbens nucleus; DL, dorsolateral striatum; DM, dorsomedial striatum; Exp. Experiment; VL, ventrolateral striatum; and VM, ventromedial striatum.

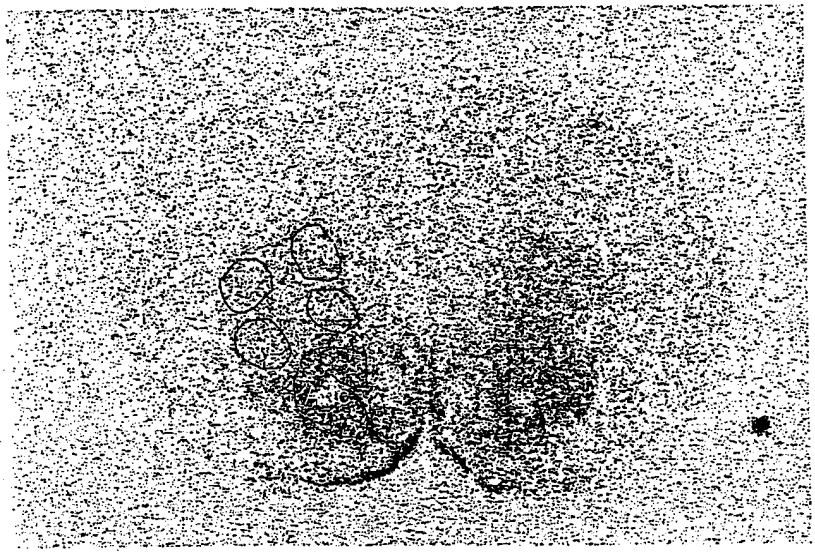


Fig. 3 Delineation of the striatal subregions in the unilaterally 6-OHDA lesioned rats, in which IGS1 mRNA ISH signals were differentially quantified.
Abbreviations: Acc, accumbens nucleus; DL, dorsolateral striatum; DM, dorsomedial striatum; VL, ventrolateral striatum; and VM, ventromedial striatum.

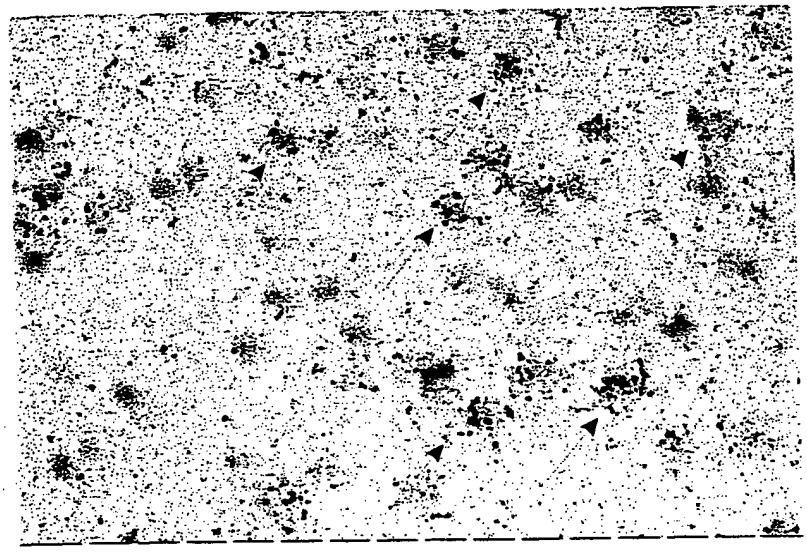


Fig. 4 IGS1 mRNA ISH labeling in the rat striatum. ISH signals are present in the medium-sized neurons (arrows), presumably dopaminergic neurons.

Rat	Section	Intact	Lesioned	Rat	Section	Intact	Lesioned	Rat	Section	Intact	Lesioned
2	79-2	120	177	5	86-2	141	191	6	60-2	110	175
2	79-3	118	163	5	86-3	112	161	6	60-3	107	173
2	70-4	103	182	5	86-4	122	161	6	60-4	112	174
Mean		*114*	*174*			*125*	*171*			*110*	*174*
SD		*9,3*	*9,8*			*14,7*	*17,3*			*2,5*	*1,0*
L vs I			***+53,1%***				***+36,8%***				***+58,7%***
p			***0,0262***				***0,0058***				***0,0003***

Fig. 5 Quantification data of the proenkephalin mRNA level in the caudate-putamen of the 6-OHDA lesioned rats. Data are expressed as the mean gray value of each measurement subtracted by the mean gray value of the background The two tail paired student's t test was applied for statistical analysis.
Abbreviations: SD, standard deviation; L, lesioned side; and I, intact side.

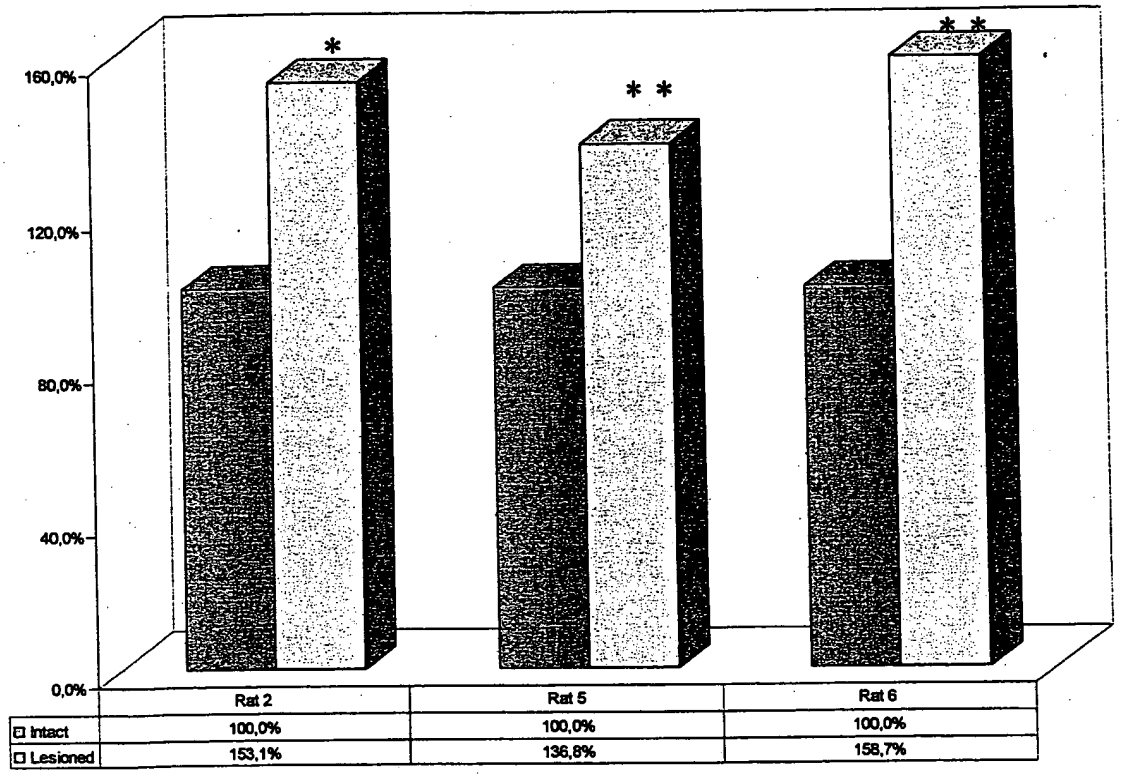


Fig. 6 Proenkephalin mRNA levels in the rat striatum after 6-OHDA lesion. Data are expressed as the percentage of the mean gray value of the intact side of each rat. * indicates $0.01 < p < 0.05$, ** $p < 0.01$.

Modulation of proenkephalin and IGS1 mRNA expressions in the rat striatum after unilateral 6-OHDA lesion

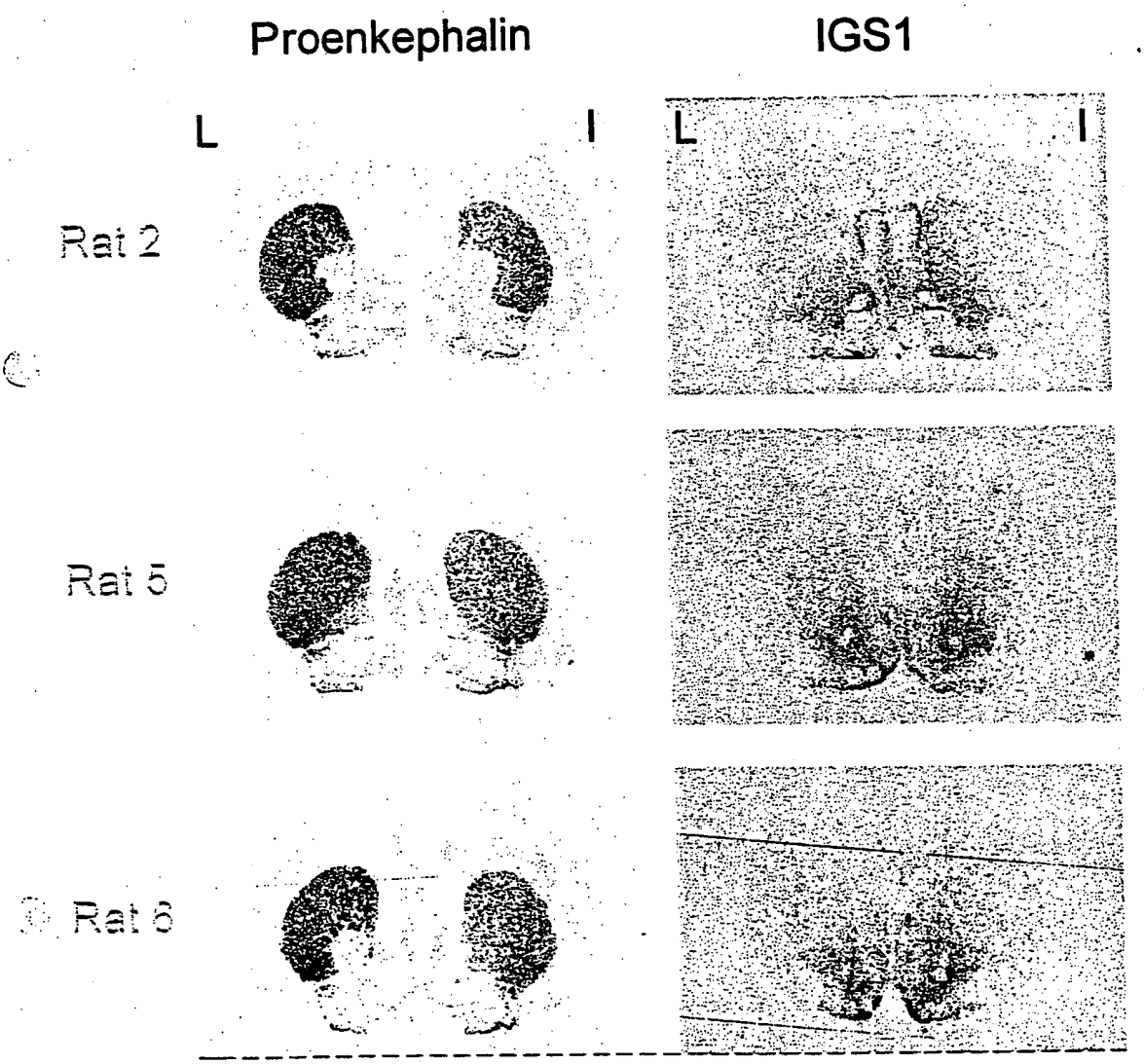
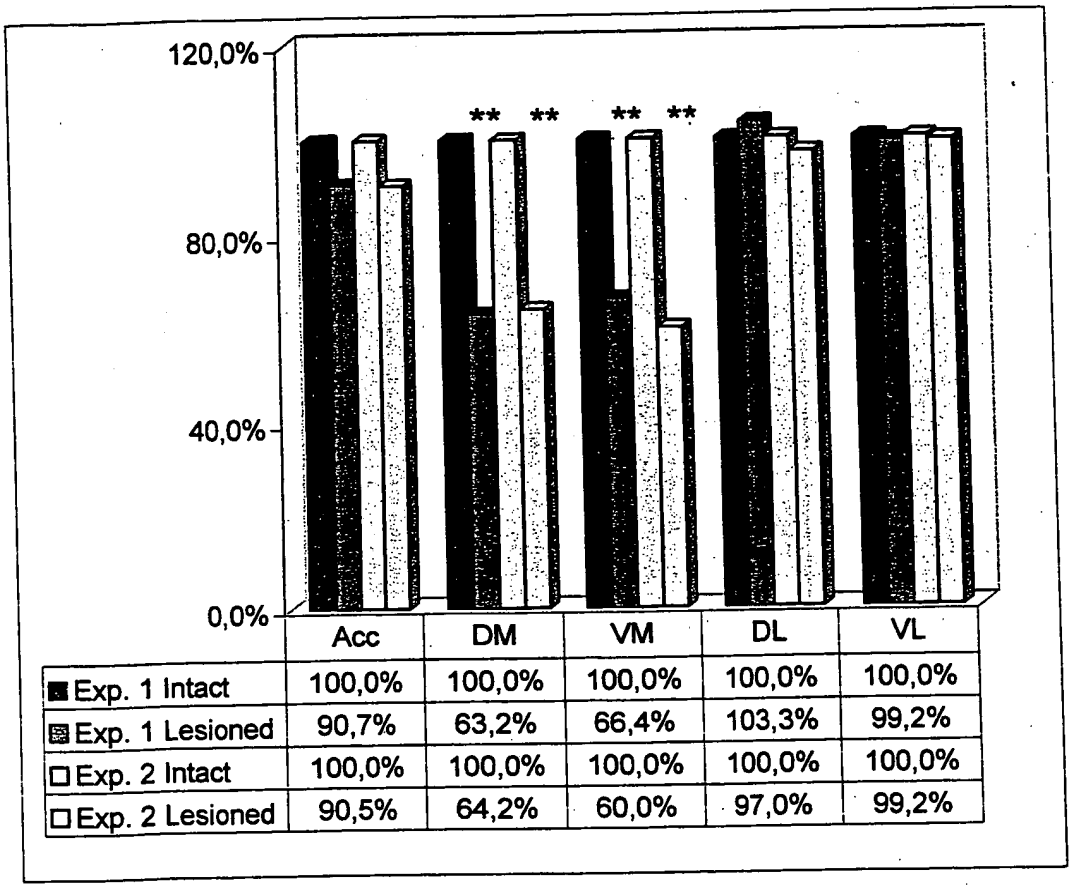


Fig. 7 An increase of ProA and a slight decrease of IGS1 mRNA levels were observed in the rat striatum ipsilateral to the 6-OHDA lesion as compared to the intact side. Abbreviations: I, intact side; L, lesioned side.

Exp. 1		Acc		DM		VM		DL		VL	
Rat	Section	Intact	Lesion	Intact	Lesion	Intact	Lesion	Intact	Lesion	Intact	Lesion
2	80-2	39.8	32.7	15.8	10.2	18.6	16.9	6.3	10.7	10.8	22.4
2	80-3	49.1	43.6	15.2	6.8	14.6	9.6	2.9	5.7	14.1	18.9
2	80-4	65.7	62.7	25.6	17.1	28.5	23.8	16.7	17.1	21.7	23.9
5	84-3	64.9	61.4	22.2	17.5	31.8	22.9	18.2	19.4	26.4	24.5
5	84-4	51.7	53.5	21.7	16.0	26.2	14.5	12.8	9.3	20.4	23.9
5	84-5	51.7	51.7	12.8	12.0	28.0	20.6	17.3	16.0	21.2	18.2
6	58-2	71.0	50.5	23.2	14.6	35.3	17.7	14.9	12.6	26.2	20.5
6	58-3	48.8	44.6	23.3	9.6	20.7	12.2	11.0	15.0	21.2	14.5
6	58-4	44.2	40.9	11.2	4.4	14.2	6.4	8.2	6.0	12.4	6.0
Mean		54.1	49.1	19.0	12.0	24.2	16.1	12.0	12.4	19.4	19.2
SD		10.6	9.7	5.2	4.6	7.5	5.9	5.3	4.9	5.7	5.9
L vs I			+ 9,3%		+ 36,8%		+ 33,6%		-3,3%		+0,8%
p			0.0462		0.0003		0.0007		0.6912		0.9383

Exp. 2		Acc		DM		VM		DL		VL	
Rat	Section	Intact	Lesion	Intact	Lesion	Intact	Lesion	Intact	Lesion	Intact	Lesion
2	79-48	79.5	66.5	32.8	23.4	48.5	25.4	18.0	24.6	29.8	31.1
2	79-49	75.5	60.7	30.4	20.0	34.9	23.6	15.6	20.7	26.2	28.5
2	79-50	82.9	70.7	39.0	23.6	43.8	31.5	24.6	22.9	33.7	28.3
5	83-48	74.4	77.6	20.7	16.0	31.3	20.5	16.1	16.5	16.0	36.5
5	83-49	*Not done, because of an artifact on the section*									
5	83-50	101.8	102.4	50.9	31.3	69.3	35.5	41.6	30.2	40.1	43.6
6	57-48	65.0	52.7	26.2	13.0	33.6	22.6	20.7	9.8	39.2	11.7
6	57-49	63.6	64.0	23.3	18.2	31.9	17.2	10.3	18.6	21.7	25.3
6	57-50	83.5	72.4	37.6	22.0	45.1	26.9	27.4	25.7	35.7	35.6
Mean		78.3	70.9	32.6	20.9	42.3	25.4	21.8	21.1	30.3	30.1
SD		12.0	14.8	9.8	5.6	12.7	5.9	9.6	6.3	8.5	9.4
L vs I			+ 9,5%		+ 35,8%		+ 40,0%		+ 3,0%		+ 0,8%
p			0.0125		0.0001		0.0002		0.7830		0.9574

Fig. 8 Quantification data of the IGS1 mRNA level in the striatum of the 6-OHDA lesioned rats. Data are expressed as the mean gray value of each measurement subtracted by the mean gray value of the adjacent background. The two tail paired Student's t test was applied for statistical analysis.
Abbreviations: Acc, accumbens nucleus; DL, dorsal lateral caudate-putamen; DM, dorsal medial caudate-putamen; VL, ventral lateral caudate-putamen; VM, ventral medial caudate-putamen.



	Acc	DM	VM	DL	VL
Exp. 1 Intact	100,0%	100,0%	100,0%	100,0%	100,0%
Exp. 1 Lesioned	90,7%	63,2%	66,4%	103,3%	99,2%
Exp. 2 Intact	100,0%	100,0%	100,0%	100,0%	100,0%
Exp. 2 Lesioned	90,5%	64,2%	60,0%	97,0%	99,2%

Fig. 9 Quantification of the IGS1 mRNA level in the striatum of the 6-OHDA lesioned rats. Data are expressed as percentage of the mean value of the intact side. Abbreviations: Acc, accumbens nucleus; DL, dorsal medial caudate-putamen; DM, dorsal medial caudate-putamen; VL, ventral lateral caudate-putamen; VM, ventral medial caudate-putamen. **$p<0.01$.

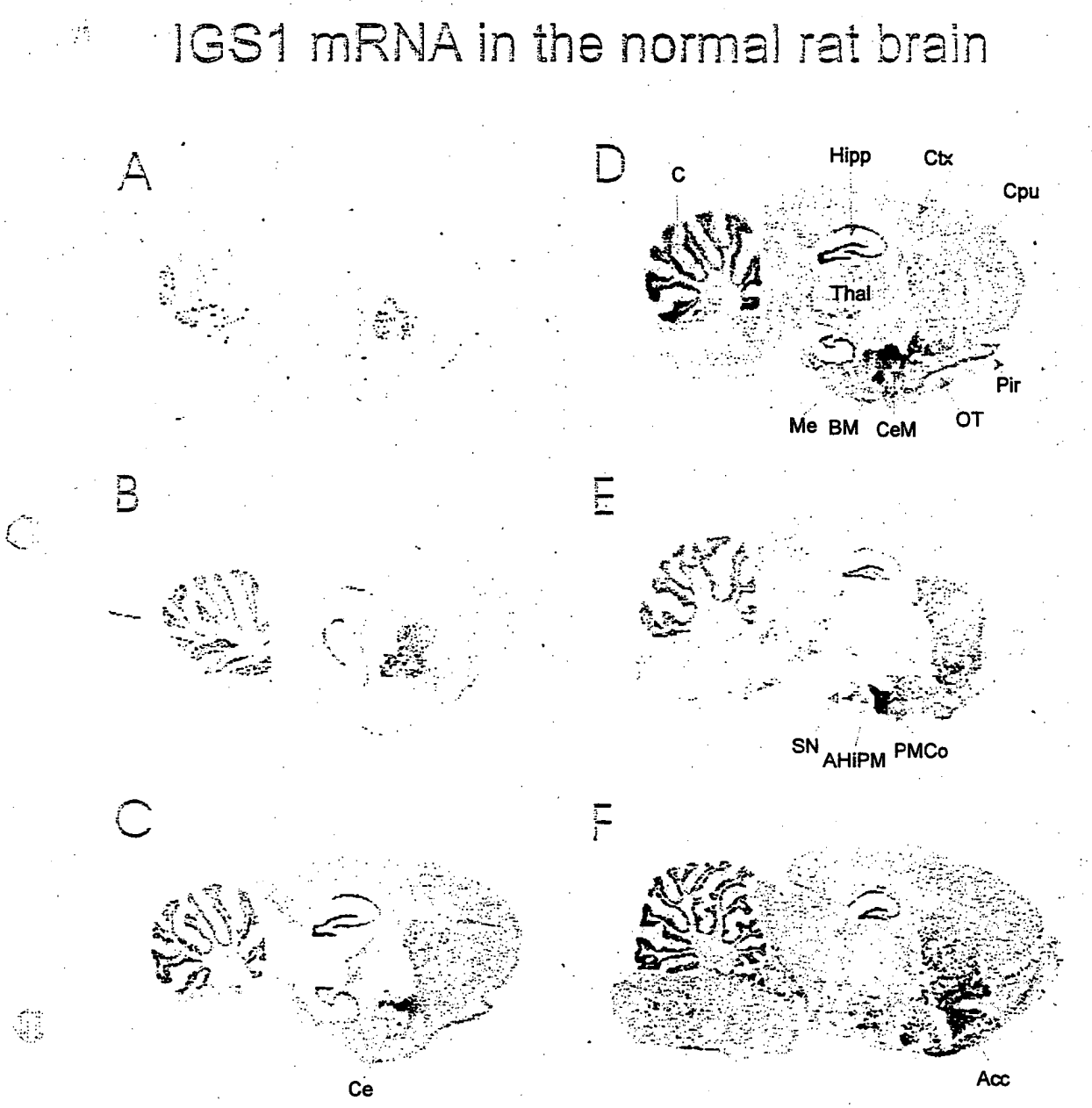


Fig. 10 Demonstration of IGS1 mRNA ISH signals in the rat sagittal sections from lateral to medial part of the normal rat brain (A to F).

Abbreviations: Acc, accumbens; AHiPM, amygdalohippocampal area posteromedial part; BM, basomedial amygdaloid nucleus; C, cerebellum; Ce, central amygdaloid nucleus; Cpu, caudate-putamen; Ctx, cerebral cortex; Hipp, hippocampus; Me, medial amygdaloid nucleus; Pir, piriform cortex; PMCo, postereomedial cortical amygdaloid nucleus; OT, olfactory tubercle; SN, substantia nigra; Thal, thalamus.